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G Protein-Coupled Estrogen Receptor 1/G Protein-Coupled Receptor 30 Localizes in the Plasma Membrane and Traffics Intracellularly on Cytokeratin Intermediate Filaments[S]

Caroline Sandén, Stefan Broselid, Louise Cornmark, Krister Andersson, Joanna Daszkiewicz-Nilsson, Ulrika E. A. Mårtensson, Björn Olde, and L. M. Fredrik Leeb-Lundberg

Department of Experimental Medical Science, Lund University, Lund, Sweden

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ABSTRACT

G protein-coupled receptor 30 [G protein-coupled estrogen receptor 1 (GPER1)], has been introduced as a membrane estrogen receptor and a candidate cancer biomarker and therapeutic target. However, several questions surround the subcellular localization and signaling of this receptor. In native cells, including mouse myoblast C_2C_{12} cells, Madin-Darby canine kidney epithelial cells, and human ductal breast epithelial tumor T47-D cells, G-1, a GPER1 agonist, and 17β-estradiol stimulated GPER1-dependent cAMP production, a defined plasma membrane (PM) event, and recruitment of β-arrestin2 to the PM. Staining of fixed and live cells showed that GPER1 was localized both in the PM and on intracellular structures. One such intracellular structure was identified as cytokeratin (CK) intermediate filaments, including those composed of CK7 and CK8, but apparently not endoplasmic reticulum, Golgi, or microtubules. Reciprocal coimmunoprecipitation of GPER1 and CKs confirmed an association of these proteins. Live staining also showed that the PM receptors constitutively internalize apparently to reach CK filaments. Receptor localization was supported using FLAG- and hemagglutinin-tagged GPER1. We conclude that GPER1-mediated stimulation of cAMP production and β-arrestin2 recruitment occur in the PM. Furthermore, the PM receptors constitutively internalize and localize intracellularly on CK. This is the first observation that a G protein-coupled receptor is capable of associating with intermediate filaments, which may be important for GPER1 regulation in epithelial cells and the relationship of this receptor to cancer.

Introduction

G protein-coupled receptor 30 (GPER1) is a GPCR that has been proposed to be an estrogen receptor responsible for at least some nongenomic estrogen signaling (Filardo and Thomas, 2005; Prossnitz et al., 2008). Estrogens are important sex hormones in both genders that have long been recognized to act through both genomic and nongenomic mechanisms. The genomic mechanisms are the best described and involve the binding of estrogens to two nuclear estrogen receptors, ERα and ERβ, which function as nuclear transcription factors regulating gene expression (Heldring et al., 2007). Membrane-associated full-length ERα also exists that is at least in part responsible for nongenomic estrogen signaling (Razandi et al., 2004).

GPER1 is ubiquitously expressed in both human and rodents, and GPER1-deficient mice show that this receptor may participate in metabolic, cardiovascular, bone, and immune regulation, at least in part, in an estrogen-dependent manner (Mårtensson et al., 2009; Olde and Leeb-Lundberg, 2009; Windahl et al., 2009). In addition, GPER1 was found to be associated with the growth of both breast and endometrial

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ABBREVIATIONS: GPER1, G protein-coupled estrogen receptor 1; B2R, B2 bradykinin receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; CK, cytokeratin; E2, 17β-estradiol; ER, endoplasmic reticulum; PM, plasma membrane; HEK, human embryonic kidney; MDCK, Madin-Darby canine kidney; GPCR, G protein-coupled receptor; FBS, fetal bovine serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PNGase F, peptide:*N*-glycanase; HA, hemagglutinin; ATCC, American Type Culture Collection; GFP, green fluorescent protein; siRNA, short interfering RNA.

cancers (Filardo et al., 2006; Smith et al., 2007). An agonist, G-1, with selectivity for GPER1 over ERα and ERβ, was reported (Bologa et al., 2006) and is now being used extensively to study this receptor. Furthermore, some antiestrogens (e.g., hydroxytamoxifen) act as agonists at this receptor (Maggiolini et al., 2004; Thomas et al., 2005).

At the cellular level, GPER1 was reported to bind E2 with high affinity (Revankar et al., 2005; Thomas et al., 2005) to influence growth factor signaling pathways, including transactivation of the epidermal growth factor receptor, intracellular Ca^{2+} mobilization, phosphatidylinositol 3-kinase translocation, Src activation, extracellular signal-regulated kinase activation, and cAMP production (Filardo and Thomas, 2005; Prossnitz et al., 2008) and to modulate downstream transcription factor networks (Pandey et al., 2009). GPER1 was antiproliferative in ERα- and ERβ-positive MCF-7 breast cancer cells but proliferative in ERα- and ERβ-negative SkBr3 breast cancer cells (Ariazi et al., 2010), suggesting that GPER1 function depends on the genetic environment of the cell.

Limited detailed studies have been done on the subcellular localization and membrane trafficking of GPER1, and then mostly in recombinant cells. Based on available studies, the localization of the receptor and receptor signaling is in debate, with some groups stating that the receptor is present and functions exclusively intracellularly in the ER either with (Revankar et al., 2005) or without estrogen receptor functions (Otto et al., 2008), whereas others state that GPER1 is present and acts as an estrogen receptor in the PM (Funakoshi et al., 2006; Filardo et al., 2007), as would be expected of a typical GPCR.

Here, we used several native cell lines to show that GPER1 is functional and localizes in the PM and intracellularly on CK intermediate filaments. CKs are proteins important for the structural integrity primarily of epithelial cells. The human genome contains a total of 54 functional CK genes, of which 37 are epithelial (Schweizer et al., 2006; Moll et al., 2008). Filamentous CK structures form by heteromeric pairing of acidic type I and basic or neutral type II CKs. Little is still known about CK beyond structural roles, but evidence is accumulating that CK may also serve additional roles as signaling platforms in cell adhesion, apoptosis/survival, and proliferation (Eriksson et al., 2009).

Materials and Methods

Cell Culture and DNA Constructs. C_2C_{12} cells, MDCK cells, and HeLa cells (ATCC, Manassas, VA) were grown in phenol-free Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) in 5% CO_2 at 37°C. T47-D cells (ATCC) were grown in RPMI 1640 media supplemented with 10% FBS and 10 μg/ml insulin in 5% CO_2 at 37°C. HEK293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS in 10% CO_2 at 37°C. The human GPER1 cDNA was subcloned into the pIRESpuro vector (Clontech, Mountain View, CA) containing a puromycin selection marker. The mouse GPER1 cDNA and human B2 bradykinin receptor (B2R) were subcloned into a pcDNA3.1 vector containing a zeosin selection marker. An N-terminal artificial signal sequence, as described previously (Whistler et al., 2002; Enquist et al., 2007), and the FLAG sequence tag were added in series to make the GPER1 construct FGPER1 and B2R construct FB2R. To make mouse GPER1 antisense cDNA, the mouse GPER1 sequence was amplified from a plasmid by PCR using the following primers: upper, 5′-CAAGCGGCCGCTATGGATGCGACTACTCCAGC-3′, and lower, 5′-CAGAAGCTTAGCACTGCTGAACCTGACCT-3′ containing a NotI and a HindIII site, respectively. The insert was then cloned in reverse orientation into the NotI/HindIII site of the pEAK12 vector. Clones containing mouse GPER1, in reverse orientation, were identified by HindIII/NotI digestion and sequencing using the BigDye terminator sequencing kit (PerkinElmer Life and Analytical Sciences, Waltham, MA). A cDNA construct of GPER1 tagged in the N terminus with three HA epitopes in series (HGPER1) was obtained from Missouri S&T cDNA Resource Center (Rolla, MO). A β-arrestin2-GFP cDNA construct was kindly provided by Dr. Marc Caron (Duke University Medical Center, Durham, NC) (Barak et al., 1997).

HEK293 cells were transfected with FGPER1 and FB2R cDNA using the calcium phosphate precipitate method and HeLa cells with human GPER1 by electroporation as described previously (Kotarsky et al., 2001). Single colonies were then chosen and propagated in the presence of selection-containing media to generate clonal stable cell lines. C_2C_{12} cells, MDCK cells, and/or T47-D cells were transiently transfected with antisense GPER1 cDNA, β-arrestin2-GFP cDNA, FGPER1 cDNA, HGPER1 cDNA, and/or GPER1 siRNA using Lipofectamine and Lipofectamine PLUS (Invitrogen) or FuGENE-6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

RNA Isolation and PCR. Isolation of RNA was performed using a method described previously (Chomczynski and Sacchi, 1987). cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System for PCR (Invitrogen). The GPER1 cDNA was amplified using PCR with the following GPER1 primers: upper, 5′-TGGCTTTGTGGGCAACATCC-3′, and lower, 5′-GGTGCTTGGTGCGGAAGAGGC-3′ (mouse); upper, 5′-TCTACACCATCTTCCTCTTCC-3′, and lower, 5′-GTAGCGATCAAAGCTCATCC-3′ (canine and human); the products were visualized on a 0.8% agarose gel.

Immunoprecipitation and Immunoblotting. Confluent cells grown on 10-cm dishes were washed twice with ice-cold PBS and lysed in 0.5 to 1 ml of lysis buffer (0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 25 mM KCl) with complete protease inhibitor cocktail (Roche Diagnostics). Lysates were cleared by centrifugation at 10,000*g* for 10 min at 4°C. Receptors were immunoprecipitated by incubating the cleared lysates overnight at 4°C with goat anti-GPER1 antibody (R&D Systems, Minneapolis, MN) coupled to protein G-Sepharose (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) or mouse anti-M2 FLAG agarose (Sigma-Aldrich, St. Louis, MO) overnight at 4°C, and CKs were immunoprecipitated with mouse anti-pan CK (clone C-11), mouse anti-CK7, or mouse anti-CK8 antibody coupled to protein G-Sepharose (all from Sigma-Aldrich). The precipitate was washed extensively and sequentially in the lysis buffer and in 10 mM Tris-HCl, pH 7.4. For immunoblotting, proteins were denatured in SDS-polyacrylamide gel electrophoresis sample buffer including 6% β-mercaptoethanol for 30 min at 37°C, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and the membrane was blocked for at least 45 min in Tris-buffered saline and 10% nonfat milk. The proteins were stained by incubating with goat anti-GPER1 antibody (1:200), mouse anti-M2 FLAG antibody (1:1000; Sigma-Aldrich), mouse HA.11 antibody (1:1000; Biosite Inc., San Diego, CA), mouse anti-pan CK antibody (1:1000), mouse anti-CK7 antibody (1:200), or mouse anti-CK8 antibody (1:200) for 1 h at 22°C. Immunoreactive bands were visualized with a chemiluminescence immunodetection kit using peroxidase-labeled secondary antibody (Invitrogen, Carlsbad, CA) according to the procedure described by the supplier (PerkinElmer Life and Analytical Sciences).

Enzymatic Deglycosylation. To determine the presence of N-glycosylation in FGPER1, immunoprecipitates were treated with 500 units of PNGase F (New England Biolabs, Ipswich, MA) in 10 mM Tris-HCl, pH 7.4, for 2 h at 37°C.

Immunofluorescence Microscopy. Cells were propagated to approximately 50% confluence in growth media on glass coverslips, coated with poly(D-lysine) or 0.1% gelatin (both from Sigma-Aldrich), and then incubated in serum- and phenol-free media for at least 1 h at 37°C before treatment. For live cell staining, live cells were incubated in serum- and phenol-free media containing goat anti-GPER1 antibody (1:100) or mouse anti-M1 FLAG antibody (1:500; Sigma-Aldrich) for 30 min at 37°C. In some experiments, live cells were treated with 0.4 M sucrose for 60 min at 37°C before incubation with antibody to disrupt clathrin-mediated endocytosis (Heuser and Anderson, 1989). Cells were then fixed with 3.7% formaldehyde in PBS and permeabilized with blotto (3% dry milk, 0.1% Triton X-100, 1 mM $CaCl_2$, and 50 mM Tris-HCl, pH 7.4). For fixed cell staining, cells were incubated in serum- and phenol-free medium and then fixed and permeabilized. The cells were then incubated in blotto containing goat anti-GPER1 antibody (1:100), mouse anti-M1 FLAG antibody (1:500), and/or mouse HA.11 antibody (1:1000) for 1 h at 22°C. In all experiments, cells were then washed with PBS and receptors visualized by incubation with secondary Alexa488-labeled anti-goat antibody, anti-mouse IgG2b antibody (Invitrogen), or anti-mouse IgG1 antibody (Invitrogen). For colocalization studies, fixed and permeabilized cells were also incubated for 1 h at 22°C with rabbit anti-calnexin antibody (1:200; Sigma-Aldrich), mouse anti-GM130 antibody (1:250; BD Biosciences, San Jose, CA), mouse anti-α-tubulin antibody (1:4000; Sigma-Aldrich), mouse pan-CK antibody (1:1000), or mouse anti-CK7 antibody (1:200). Alexa568-labeled anti-mouse IgG1 or anti-rabbit antibody (Invitrogen) were then used as secondary antibodies. For β-arrestin2-GFP imaging, cells were incubated in serum- and phenol-free with or without 1 μM E2 (Sigma-Aldrich), 1 μM G-1 (Calbiochem, San Diego, CA), 1 μM isoproterenol (Sigma-Aldrich), or DMSO vehicle for 30 min at 37°C and then fixed with 3.7% formaldehyde in PBS and washed with PBS. Images were collected using a Nikon Eclipse confocal fluorescence microscope (Nikon, Tokyo, Japan). Some fluorescence images were analyzed using NIS Elements software (Nikon) and then graphed.

cAMP Production. Cells were grown to near confluence in six-well plates (Sarstedt, Nümbrecht, Germany). The cells were washed one time with serum- and phenol-free medium followed by incubation in the same medium for 1 h at 37°C. This was followed by 20 min of incubation at 37°C in medium containing 25 μM rolipram (Sigma-Aldrich). Different concentrations of E2 and G-1 were added, and the cells were further incubated for 30 min, after which the cells were lysed in 0.20 ml of ice-cold 0.1 M HCl for 30 min at 4°C, scraped, and centrifuged at 13,000*g* for 10 min. The amount of cAMP in the supernatant was assayed using an EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Data Analysis. Data are presented as means ± S.E.M. Student's two-tailed *t* test for unpaired data were performed to evaluate statistical significance. *P* values less than 0.05 were regarded as statis-

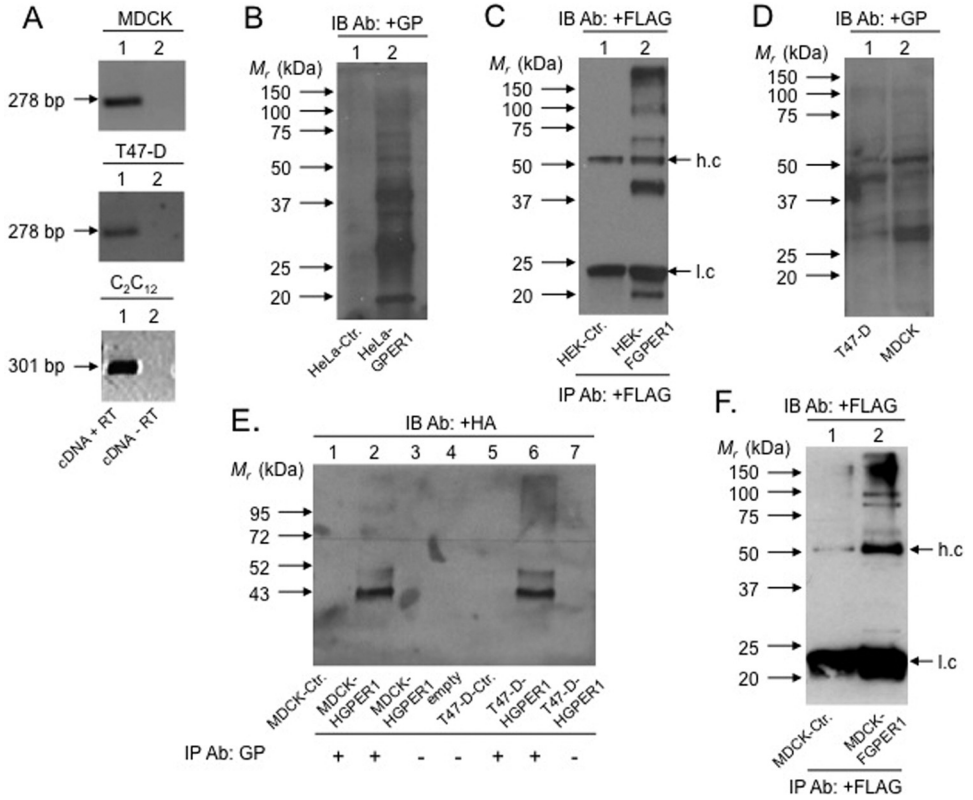

Fig. 1. GPER1 expression. A, RNA from MDCK cells, T47-D cells, and C_2C_{12} cells was isolated and cDNA synthesized and analyzed with (cDNA+RT, lane 1) and without reverse transcriptase (cDNA-RT, lane 2). B, HeLa cells without (HeLa-Ctr., lane 1) and with stable expression of human GPER1 (HeLa-GPER1, lane 2) were lysed and immunoblotted with goat GPER1 antibody (GP). C, HEK293 cells without (HEK-Ctr., lane 1) and with stable expression of mouse FGPER1 (HEK-FGPER1, lane 2) were lysed, immunoprecipitated with mouse M2 FLAG antibody-agarose, and immunoblotted with M2 FLAG antibody (FLAG). D, T47-D cells (T47-D, lane 1) and MDCK cells (MDCK, lane 2) were lysed and immunoblotted with GPER1 antibody (GP). E, mock-transfected MDCK cells (MDCK-Ctr., lane 1) and T47-D cells (T47-D-Ctr., lane 5) and MDCK cells (MDCK-HGPER1, lanes 2 and 3) and T47-D cells (T47-D-HGPER1, lanes 6 and 7) transiently transfected with HGPER1 cDNA were lysed, immunoprecipitated with protein G-Sepharose without (−, lanes 3 and 7) and with GPER1 antibody (GP) (+, lanes 2 and 3), and immunoblotted with HA antibody. F, mock-transfected MDCK cells (MDCK-Ctr., lane 1) and MDCK cells transiently transfected with FGPER1 cDNA (MDCK-FGPER1, lane 2) were lysed, immunoprecipitated with M2 FLAG antibody beads, and immunoblotted with M2 FLAG antibody. Molecular mass (M_r) standards (in kilodaltons), or base pairs (bp) (left side arrows), and position of IgG heavy chain (h.c.) and light chain (l.c.) (right side arrows) are indicated. The results are representative of experiments performed at least three times.

tically significant. Data analysis was performed using the Prism program (GraphPad Software Inc., San Diego, CA).

Results

GPER1 Expression. Several native cell systems relevant to GPER1 physiology from several species were used to study GPER1, including canine kidney epithelial MDCK cells, mouse myoblast C_2C_{12} cells, and human ductal breast epithelial tumor T47-D cells. All of the cells expressed GPER1 as determined at the mRNA level (Fig. 1A). Receptor expression at the protein level was monitored with a goat anti-GPER1 antibody raised against the human receptor N-terminal domain, which was used previously to detect GPER1 (Kolkova et al., 2010). To verify that this antibody reacts specifically with GPER1, we immunoblotted control HeLa cells (HeLa-Ctr.) (Fig. 1B, lane 1) and cells stably expressing human GPER1 (HeLa-GPER1) (lane 2). No bands were observed in HeLa-Ctr. cells, whereas major bands were observed in HeLa-GPER1 cells. The 40- to 45-kDa band corresponds most closely with the theoretical mass of the receptor, whereas the lower bands (20 and 30 kDa) are probably degradation products thereof. To further evaluate receptor expression, control HEK293 cells (HEK-Ctr.) (Fig. 1C, lane 1) and cells stably expressing mouse GPER1 with the FLAG epitope inserted at the N-terminal end (FGPER1) (HEK-FGPER1) (lane 2) were immunoprecipitated with M2 FLAG antibody covalently coupled to agarose and immunoblotted with FLAG antibody. A receptor band at 40 to 45 kDa was present also in HEK-FGPER1 cells as well as a band at 20 kDa. In addition, these cells contained receptor bands at 65 and 100 kDa and greater that may be receptor complexes.

Immunoblotting of T47-D cell (Fig. 1D, lane 1) and MDCK cell lysates (lane 2) with GPER1 antibody revealed bands common to these cells at approximately 40 to 45 kDa as well as at 30 and 50 to 55 kDa, albeit with slightly different relative intensities. The GPER1 antibody did not recognize the mouse receptor as determined with both mouse C_2C_{12} cells and HEK-FGPER1 cells. Thus, to further evaluate the GPER1 antibody specificity, T47-D cells and MDCK cells were transfected with or without a cDNA of human GPER1 containing the HA epitope at the N-terminal end (HGPER1) and then immunoprecipitated with protein G-Sepharose with and without precoupled GPER1 antibody. As shown in Fig. 1E (lanes 2 and 6), the GPER1 antibody recognized HGPER1 bands at 40 to 45 and 50 to 55 kDa as well as a weak band at approximately 100 kDa in both T47-D-HGPER1 cells and MDCK-HGPER1 cells that were absent in mock-transfected T47-D-Ctr. and MDCK-Ctr. cells (lanes 1 and 5) and immunoprecipitates with only protein G-Sepharose (lanes 3 and 7). In MDCK cells transfected with FGPER1 cDNA (MDCK-FGPER1), FGPER1-specific bands were present at 50 to 55 kDa, in part overlapping with the IgG heavy chain, and at approximately 65 and 90 to 100 kDa (Fig. 1F, lane 2) that were absent in mock-transfected MDCK- Ctr. cells (Fig. 1F, lane 1). Thus, FGPER1 migrates in part differently in MDCK and HEK293 cells with a band at 40 to 45 kDa in the former cells and a band at 50- to 55-kDa band in the latter cells. However, the presence of a 50- to 55-kDa band also in HEK293 cells was indicated after PNGase deglycosylation (see below). On the other hand, HGPER1 clearly migrates at both masses. Thus, GPER1 seems to reside on proteins of 40 to 45 and 50 to 55 kDa as determined with GPER1 antibody, which reacts with the native receptor, FLAG antibody, which reacts with FGPER1, and HA antibody, which reacts with HGPER1. Receptor immunoprecipitates also enriched for a higher mass receptor form(s) at approximately 100 kDa, which may be a detergent-resistant receptor complex(es). Together, these results show that the GPER1 antibody is specific for GPER1.

GPER1 N-Glycosylation. The presence of multiple immunoreactive GPCR species is typical and often caused by variations in receptor N-glycosylation. To address this modification as a basis for GPER1 heterogeneity, FLAG immunoprecipitates from HEK-FGPER1 cell lysates were N-deglycosylated with PNGase F and then immunoblotted with FLAG antibodies. Surprisingly, PNGase F treatment resulted in a dramatic decrease in the intensities of the 20-, 40- to 45-, and 65-kDa bands and an increase in the intensity of the 100-kDa band (Fig. 2A), which is contrary to the typical decrease in apparent receptor mass that occurs upon deglycosylation. A decrease did occur in the mass of a FLAG-specific protein corresponding to that of the IgG heavy chain (50–55 kDa) supporting the presence of a receptor band here also in these cells. Similar changes occurred upon PNGase F treatment of FGPER1 immunoprecipitates from lysates of MDCK-FGPER1 cells and T47-D-FGPER1 cells (Fig. 2B), again with the notable decrease in mass at 50 to 55 kDa. These results show that the receptor is N-glycosylated and that this modification has additional effects on the physical properties of the receptor, at least FGPER1.

GPER1-Mediated cAMP Production. G-1, a substance reported previously to be an agonist on GPER1-mediated intracellular Ca^{2+} signaling (Bologa et al., 2006), dose-dependently increased cAMP production in a saturable manner in

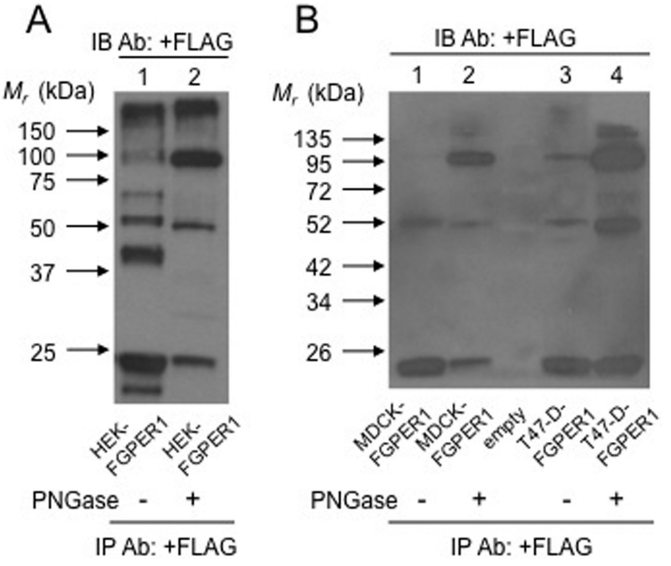

Fig. 2. GPER1 N-glycosylation. A, HEK293 cells stably expressing mouse FGPER1 (HEK-FGPER1) were lysed, immunoprecipitated with mouse M2 FLAG antibody-agarose, treated without (lane 1) and with PNGase F (lane 2), and immunoblotted with M2 FLAG antibody. B, MDCK cells (MDCK-FGPER1, lanes 1 and 2) and T47-D cells (T47-D-FGPER1, lanes 3 and 4) transiently transfected with FGPER1 cDNA were lysed, immunoprecipitated with M2 FLAG antibody beads, treated without (lanes 1 and 3) and with PNGase F (lanes 2 and 4), and immunoblotted with M2 FLAG antibody. Molecular mass standards (in kilodaltons) (left side arrows) are indicated. The results are representative of experiments performed at least three times.

mouse C_2C_{12} cells with an EC_{50} value of 282 ± 52 nM (Fig. 3A). E2 also potently stimulated cAMP production in these cells with an EC_{50} value of 1.8 ± 0.3 nM (Fig. 3A). However, the maximal response, or efficacy, of E2 was only approximately 10% of that of G-1. To address the dependence of these responses on GPER1, we transiently transfected C_2C_{12} cells with a mouse antisense GPER1 cDNA construct used previously and validated in detail (Ahola et al., 2002; Revankar et al., 2005). The G-1- (Fig. 3B) and E2-promoted responses (Fig. 3C) were both inhibited by this antisense construct. G-1 and E2 also stimulated cAMP production in MDCK cells with relative efficacies and potencies similar to those in C_2C_{12} cells (Fig. 3D). A canine GPER1-specific siRNA, but not a scrambled nonspecific siRNA, inhibited the G-1 response in MDCK cells (Fig. 3E), indicating that this response was also dependent on GPER1. G-1 also stimulated cAMP production in human T47-D cells (Fig. 3F), which express GPER1 (Fig. 1A). Thus, G-1- and E2-stimulated cAMP production in these native cells is mediated at least in part by GPER1.

GPER1-Mediated β-Arrestin2 Recruitment. Receptor-mediated cAMP production is a PM-dependent event. To further address the subcellular localization of GPER1 signaling, we analyzed the distribution of β-arrestin2, a regulatory and signaling effector protein that physically associates with many GPCRs at their site of function. Consistent with cAMP signaling, G-1 and E2 increased β-arrestin2-GFP in the PM in MDCK cells (Fig. 4). Isoproterenol was used as a positive control presumably by acting through a small but significant number of β_2-adrenergic receptors expressed on these cells (Meier et al., 1983). PM recruitment of β-arrestin2-GFP by these agents was also observed in T47-D cells (data not shown). These results provide additional evidence that GPER1 functions at least in part in the PM. Even though only semiquantitative, this assay further suggests that G-1 and E2 exhibit similar efficacies on this response.

Subcellular GPER1 Trafficking. Limited detailed studies have been done to localize GPER1 subcellularly in native cells. To do so, we performed confocal immunofluorescence microscopy using the GPER1 antibody. The dependence of antibody reactivity on GPER1 expression was again confirmed by positive staining in HeLa-GPER1 cells but not in HeLa-Control cells (Fig. 5A). A calnexin antibody was used as a control to show ER staining in both cells types. Some overlap in receptor and ER staining occurred, which is common in overexpressed recombinant GPCR cell systems and is often due to saturation of maturation mechanisms (Fig. 5A).

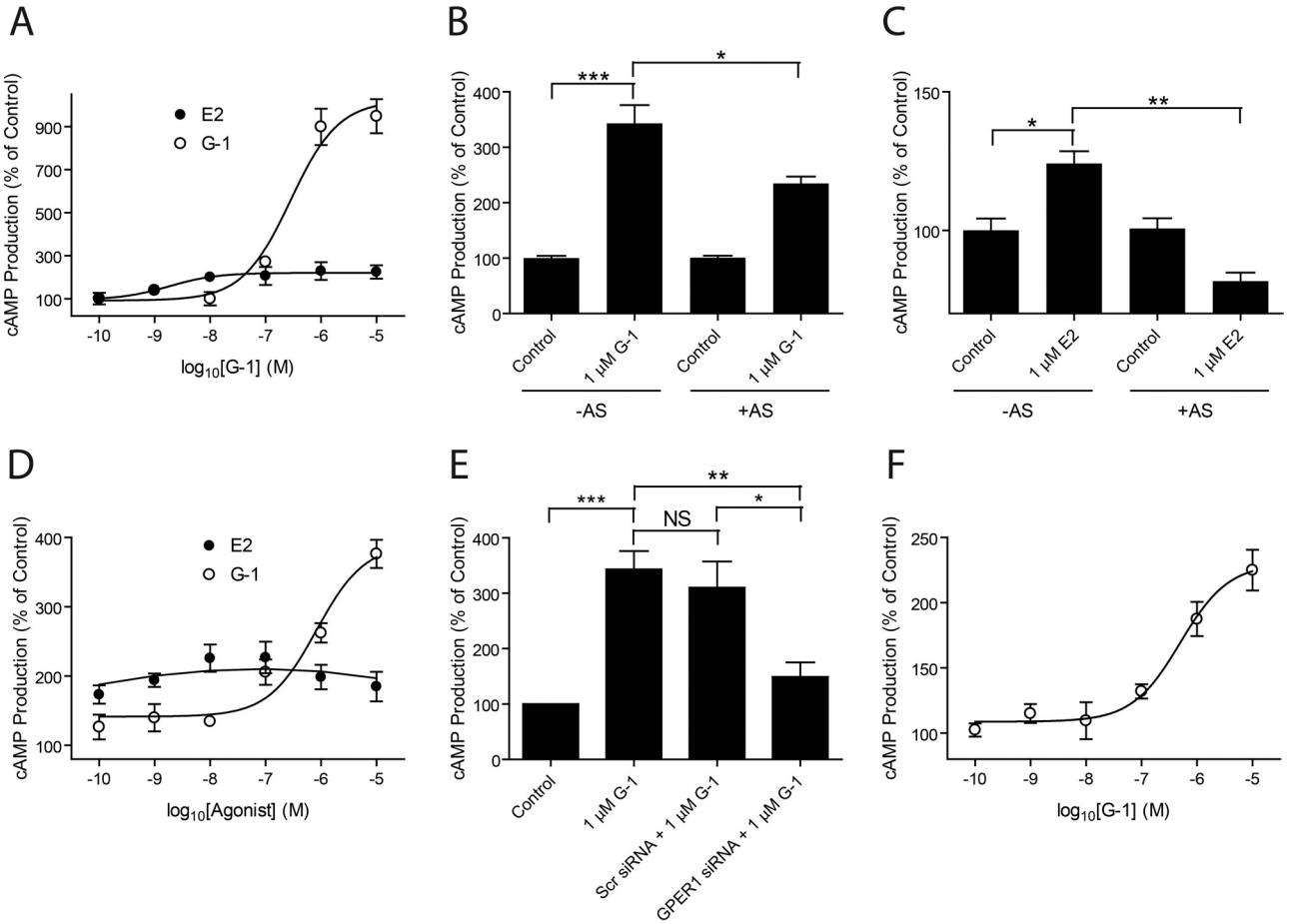

Fig. 3. GPER1-mediated cAMP production. A, C_2C_{12} cells were stimulated with increasing concentrations of G-1 (○) or E2 (●) for 30 min at 37°C and then assayed for cAMP production. B and C, C_2C_{12} cells were transfected with a mouse GPER1 antisense construct (+AS) or empty vector (−AS), stimulated without (Control) or with 1 μM G-1 (B) or 1 μM E2 (C) for 30 min at 37°C and then assayed for cAMP production. D, MDCK cells were stimulated with increasing concentrations of G-1 or E2 for 30 min at 37°C and then assayed for cAMP production. E, MDCK cells were transfected with a canine GPER1 siRNA (GPER1 siRNA) or scrambled siRNA (Scr siRNA) construct, stimulated without (Control) or with 1 μM G-1 for 30 min at 37°C, and then assayed for cAMP production. F, T47-D cells were stimulated with increasing concentrations of G-1 and then assayed for cAMP production. Data are presented as a percentage of control where control corresponds to 15 to 20 pmol cAMP/well. Values are means ± S.E.M. with each data point performed in quadruplicate. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The specificity of the antibody for GPER1 was further underlined by colocalization of GPER1 antibody and FLAG antibody staining in T47-D cells transfected with human GPER1 and FGPER1 (Fig. 5B). Furthermore, FLAG antibody and HA antibody staining colocalized in cells transfected with FGPER1 and HGPER1 (Fig. 5B). Costaining of GPER1 and HGPER1 with the GPER1 antibody could not be done because both receptor constructs are of human origin and are thus recognized by this antibody. It is noteworthy that the intracellular costaining had a web-like appearance in all cells, suggesting receptor localization on a cytoskeletal structure.

Staining of endogenous GPER1 with the GPER1 antibody in fixed MDCK cells was observed both at the cell periphery and intracellularly, the latter also exhibiting a web-like cytoskeletal appearance (Fig. 5B; Supplemental Fig. 1). Staining also occurred under live conditions at 37°C, providing direct evidence for PM receptors because the antibody is directed against the extracellular N-terminal receptor domain (Fig. 5C). A significant amount of the live staining was intracellular, showing that the PM receptors undergo constitutive endocytosis (Fig. 5C). The live intracellular staining exhibited the same web-like pattern as the fixed intracellular staining (Fig. 5C), suggesting that this pattern is at least in part caused by constitutive receptor internalization.

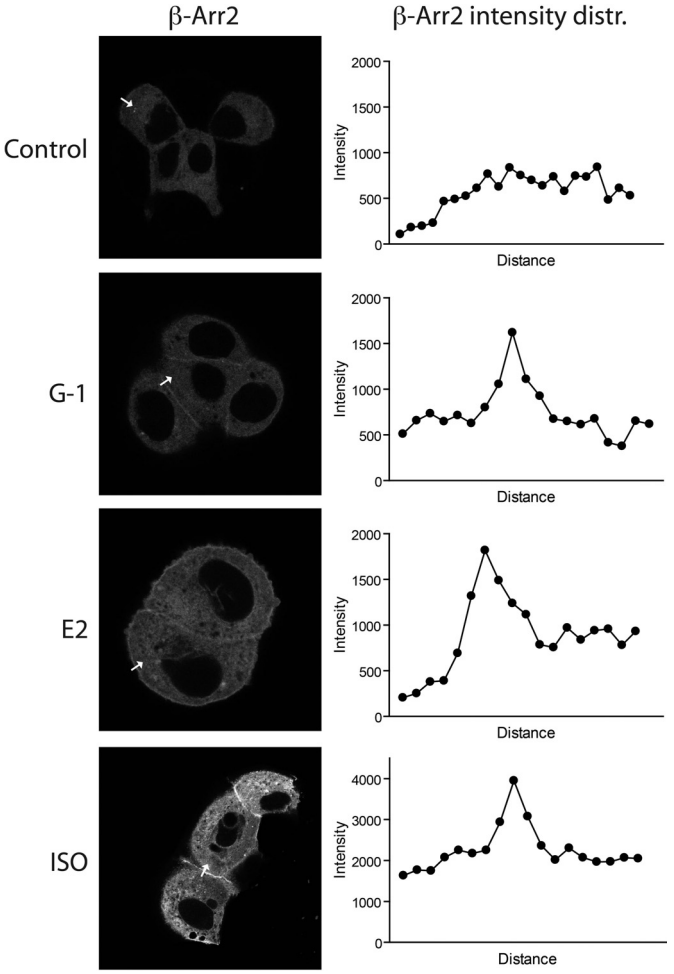

Fig. 4. GPER1-mediated β-arrestin2 recruitment. MDCK cells transiently transfected with β-arrestin2-GFP cDNA were stimulated without (Control) or with 1 μM G-1, 1 μM E2, or 1 μM isoproterenol (ISO) for 30 min at 37°C. Images of fixed cells were collected using a Nikon Eclipse confocal microscope with 60× objective and 50 μm zoom. Arrows indicate cellular distance that was analyzed for changes in β-arrestin2-GFP fluorescence intensity upon agonist stimulation using the NIS Elements software program (Nikon), and the results are graphed. Distance (arrow) is 2 μm. The results are representative of experiments performed at least three times.

To confirm constitutive GPER1 endocytosis, we used HEK-FGPER1 cells. Fixed staining with M1 FLAG antibody showed that FGPER1 was present primarily intracellularly (Fig. 5C), similar to HeLa-GPER1 cells (Fig. 5A). Live staining was again observed, indicating the presence of PM receptors also in these cells. Similar to MDCK cells, the live staining was almost exclusively intracellular, again showing constitutive receptor internalization (Fig. 5D). The live staining in HEK-FGPER1 cells was punctate rather than web-like, which again may be due to the heterologous nature of this overexpressed recombinant cell system. Incubating cells in the absence of serum for 24 h did not change the live staining pattern, showing that it was not caused by a serum-derived factor. The relative amount of cell surface receptor staining increased dramatically by treating the cells before antibody incubation with hyperosmotic sucrose for 30 min, which blocks endocytosis by yielding abnormal clathrin polymerization, resulting in empty microcages in the membrane (Heuser and Anderson, 1989) (Fig. 5D). The live intracellular staining was not caused by nonspecific uptake of the antibody or antibody-promoted receptor internalization because GPCRs vary in their ability to generate such staining as described previously by us (Enquist et al., 2007). Indeed, FLAG-tagged B2 bradykinin receptors (FB2Rs) stably expressed in HEK293 (HEK-FB2R) remained in the PM during live staining until exposed to the agonist bradykinin, upon which the receptor-antibody complex internalized (Fig. 5D). Thus, two different cell systems (MDCK cells and HEK-FGPER1) using two different receptor-specific antibodies (GPER1 antibody and M1 FLAG antibody) show that at least a fraction of the cellular GPER1 is localized in the PM and undergoes constitutive endocytosis to populate intracellular structures.

Subcellular GPER1 Localization. Antibodies against various subcellular marker proteins were then used to determine the intracellular localization of GPER1 in native cells. Intracellular GPER1 staining in MDCK cells was not associated with ER, as determined by the lack of overlap with calnexin staining (Fig. 6A). In addition, no overlap occurred with GM130 staining of Golgi (data not shown). The web-like intracellular staining pattern suggested receptor association with a cytoskeletal structure. Lack of costaining with α-tubulin staining showed that this structure is not microtubules (Fig. 6B). In addition, receptor staining seemed too disorganized to be associated directly with actin filaments. On the other hand, a significant overlap occurred in receptor and CK staining (Fig. 6C). The same overlap was observed in T47-D cells (Fig. 6C). The pan-CK antibody that was used recognizes several CKs including the simple epithelial CK8, but not CK7. Using a specific CK7 antibody, overlap was also found with this CK subtype (Fig. 6D). Thus, in MDCK cells and T47-D cells, GPER1 is localized in the PM and intracellularly at least in part on CK intermediate filaments but not in the ER, Golgi, or on microtubules.

GPER1-CK Association. GPER1-CK association was further addressed by coimmunoprecipitation. Immunoblotting of MDCK and T47-D cells with anti-pan-CK antibody re-

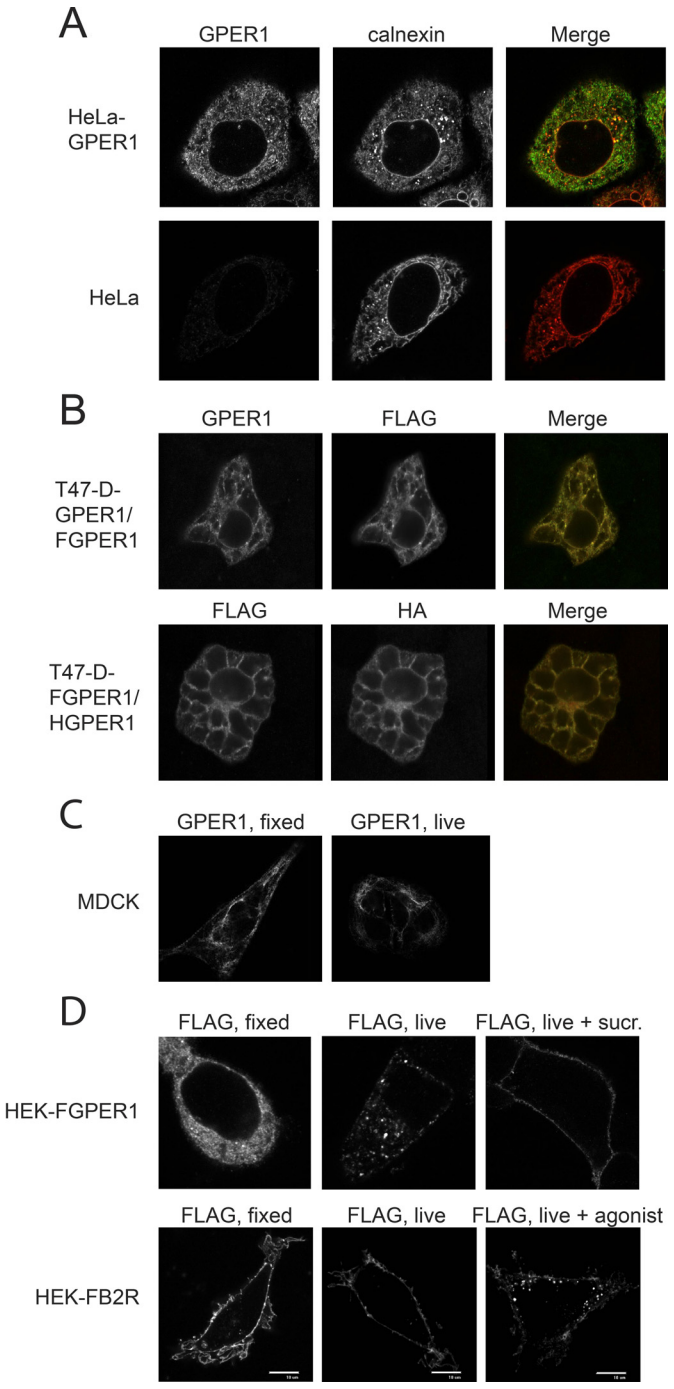

Fig. 5. Subcellular GPER1 trafficking. A, HeLa cells without (HeLa-Control) and with stable expression of human GPER1 (HeLa-GPER1) were fixed and permeabilized before incubation with goat GPER1 antibody (GPER1) and rabbit calnexin antibody (calnexin). B, T47-D cells transfected with human GPER1 and FGPER1 (T47-D-GPER1/FGPER1) or FGPER1 and HGPER1 (T47-D-FGPER1/HGPER1) were fixed and permeabilized before incubation with goat GPER1 antibody (GPER1), mouse FLAG antibody (FLAG), and/or mouse HA antibody (HA). C, MDCK cells (MDCK) were fixed and permeabilized before incubation with GPER1 antibody (GPER1, fixed) or preincubated live with GPER1 antibody for 30 min at 37°C before fixation and permeabilization (GPER1, live). D, HEK293 cells stably expressing mouse FGPER1 (HEK-FGPER1) and human FB2R (HEK-FB2R) were fixed and permeabilized before incubation with mouse M1 FLAG antibody (FGPER1, fixed; FB2R, fixed) or preincubated live without (FGPER1, live; FB2R, live) or with 0.4 M sucrose for 60 min (FGPER1, live + sucr) or 1 μM bradykinin for 30 min (FB2R, live + agonist) at 37°C before incubation with M1 FLAG antibody for an additional 30 min at 37°C. The cells were then fixed and permeabilized. In A to D, cells were subsequently incubated with secondary donkey anti-goat, rabbit anti-mouse, or mouse anti-rabbit ALEXA488- or ALEXA568-labeled antibody. The individual and merged (Merge) images were collected using a Nikon Eclipse confocal microscope, 60× objective, 50 μm zoom. The results are representative of experiments performed at least three times.

vealed that both cells express CKs at masses of 50 to 55 kDa, which is typical of epithelial CKs (Fig. 7A, lanes 1 and 2). The T47-D cell line was the richest source of CK, which is expected of a breast cancer epithelial cell line. Typical of a simple epithelial cell, T47-D cells expressed the basic type-II CKs CK8 (Fig. 7A, lane 3) and CK7 (lane 4), often as doublets, and as reported previously (Ferrero et al., 1989). Even though MDCK cells have been reported to express CK8 (Pollack et al., 1997), we were unable to effectively detect CK7 or CK8 in MDCK cell lysates, which is probably due to the relatively low CK expression in this cell line (Fig. 7A, lane 1).

Consistent with GPER1-CK association, GPER1 immunoprecipitates from both MDCK and T47-D cells contained CK (Fig. 7B, lanes 1 and 2) including both CK7 (lanes 3 and 4) and CK8 (lanes 5 and 6). CKs detected by the pan-CK antibody in both cells (Fig. 7B, lanes 1 and 2) and CK7 antibody in MDCK cells (lane 3) migrated as monomers, whereas higher mass forms were detected of CK7 in T47-D cells (lane 4) and CK8 in both cells (lanes 5 and 6). The reason for this is unknown but may be due in part to detergent-resistant CK complexes with, for example, GPER1. Indeed, pan-CK immunoprecipitates from T47-D cells contained a GPER1 antibody-reactive band at approximately 100 kDa (Fig. 7C, lane 2) that was not present in protein G-Sepharose precipitates (lane 1). The same specific band was present in CK8 and/or CK7 immunoprecipitates of MDCK cells (Fig. 7D, lanes 2 and 3) and T47-D cells (lane 4). Weaker bands at approximately 50 kDa occasionally appeared in protein G-Sepharose precipitates (Fig. 7D, lane 1). PNGase F treatment of the pan-CK immunoprecipitate from T47-D cells resulted in a small downward shift in the 100-kDa GPER1 band (Fig. 7E, lanes 1 and 2), whereas this treatment did not influence the CK band in the GPER1 immunoprecipitate (Fig. 7E, lanes 3 and 4).

FLAG immunoprecipitates of MDCK-FGPER1 cells also contained pan-CK and CK8 immunoreactivities of higher masses (Fig. 8, lanes 2 and 4) that were not present in mock-transfected MDCK-Ctr. cells (lane 1). Consistent with native cells, pan-CK immunoprecipitates from MDCK-FGPER1 cells contained a 100-kDa FLAG-receptor-specific band (Fig. 8, lane 5). Thus, the 100-kDa receptor species observed in both FGPER1 and HGPER1 immunoprecipitates seems to be a major CK-interacting partner as determined by immunoblotting with both GPER1 antibody for the native receptor (Fig. 7, C–E) and FLAG antibody for FGPER1 (Fig. 8, lane 5). In all, these results confirm those obtained by immunofluorescence microscopy that at least some GPER1 in MDCK cells and T47-D cells associate with CK intermediate filaments.

Discussion

Here, we investigated the subcellular distribution and signaling of GPER1 expressed endogenously in a series of pathophysiologically relevant cell lines from various species, including MDCK cells, T47-D cells, and C_2C_{12} cells. GPER1 was localized both in the PM and on intracellular cytoskeletal structures. The PM receptors were subject to relatively

rapid constitutive endocytosis to reach such structures. These structures were identified at least in part as CK intermediate filaments, including those composed of CK7 and CK8, but not ER, Golgi, or microtubules. The GPER1 agonist G-1 and E2 stimulated GPER1-dependent cAMP production and β-arrestin2 recruitment to the PM. We conclude from this study that GPER1 couples to cAMP and β-arrestin2 signaling in the PM and associates intracellularly with CK, the latter of which may be a mechanism for subcellular redistribution of the receptor after endocytosis.

The subcellular localization of GPER1 has been a point of debate ever since this receptor was introduced as a putative estrogen receptor in 2005. Four studies were published early that addressed this issue using epitope-tagged receptors expressed in recombinant cell systems. Three of these studies used receptors tagged at the N terminus with either the HA epitope (Thomas et al., 2005; Filardo et al., 2007) or the FLAG epitope (Funakoshi et al., 2006) to claim that the receptor localized in the PM but reached intracellular compartments via E2-promoted endocytosis. Two other studies used receptors tagged in the C terminus with GFP, in the N terminus with the FLAG epitope, or nontagged receptor to claim that the receptor is more or less exclusively localized intracellularly in the ER (Revankar et al., 2005; Otto et al., 2008). Another study found that when GPER1 was tagged at the N terminus with the FLAG epitope it localized in the PM, whereas when tagged at the C terminus with GFP, it localized in the ER (Funakoshi et al., 2006), which agrees with some other epitope-tagged GPCR (Brothers et al., 2003) and indicates that tagging can influence GPCR trafficking. Since then, several studies have addressed endogenous receptor localization using various receptor antibodies and reported immunoreactivity at the cell periphery and/or intracellularly in the ER and/or Golgi (Funakoshi et al., 2006; Brailoiu et al., 2007; Sakamoto et al., 2007; Matsuda et al., 2008; Otto et al., 2008; Lin et al., 2009). Even though not emphasized, many of these reports, however, also show significant intracellular immunoreactivity not associated with either of these structures. Only in one study was PM localization of endogenous GPER1 directly addressed. In this case, specific radiolabeled E2 binding to a PM fraction from SkBr3 breast cancer cells was detected that was sensitive to prior treatment of the cells with a GPER1 siRNA (Thomas et al., 2005).

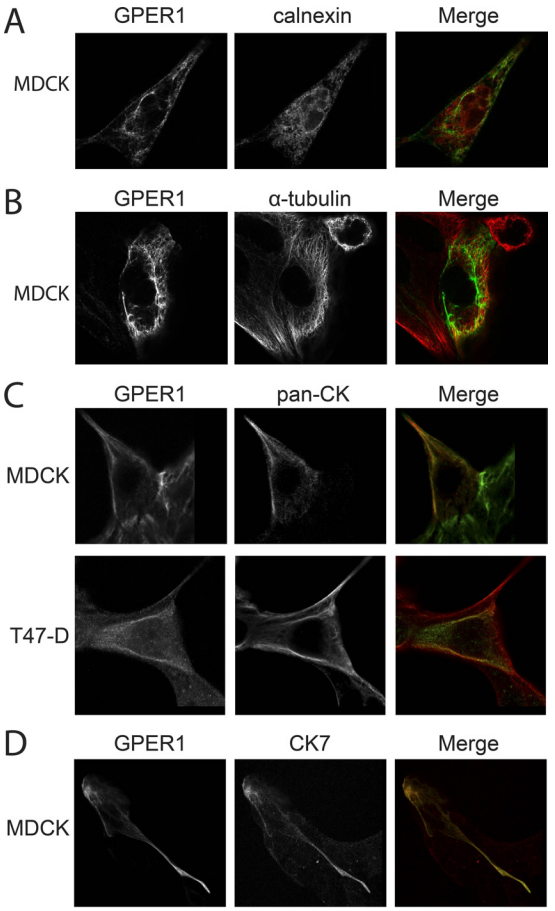

Fig. 6. Subcellular GPER1 localization. A to D, MDCK cells (MDCK) or T47-D cells (T47-D) were fixed and permeabilized before incubation with goat GPER1 antibody (GPER1), rabbit calnexin antibody (calnexin), mouse α-tubulin antibody (α-tubulin), mouse pan-CK antibody (pan-CK), or mouse CK7 antibody (CK7). Cells were subsequently incubated with secondary mouse anti-rabbit, rabbit anti-mouse, or donkey anti-goat ALEXA488- or ALEXA568-labeled antibody. The individual and merged (Merge) images were collected using a Nikon Eclipse confocal microscope, 60× objective, 50 μm zoom. The results are representative of experiments performed at least three times.

In this study, using an antibody that we confirmed to be specific for GPER1, immunostaining of fixed cells showed that endogenous GPER1 is localized both at the cell periphery and intracellularly in MDCK cells and T47-D cells. Furthermore, human GPER1, mouse FGPER1, and human HGPER1 colocalized on the same cytoskeleton-like cellular structures. MDCK cells stained positive under live nonfixed conditions, indicating that GPER1 is also present at the PM in these cells. No significant overlap occurred between receptor staining and either ER or Golgi staining, which suggests that the receptor matures normally in these cells. The ability of GPER1 to reach the PM was confirmed by live staining of HEK-FGPER1 cells with FLAG antibody. Live staining of MDCK cells and HEK-FGPER1 cells also showed that the PM receptors can reach intracellular structures via constitutive endocytosis. In MDCK cells, live and fixed staining had the same cytoskeleton-like patterns, suggesting, at least in part, that the constitutively internalized receptors give rise to such staining.

Three experimental observations led us to conclude that at least a portion of the cytoskeleton-like receptor staining represents receptors associated with CK intermediate filaments, including 1) colocalization of GPER1 and CK staining as determined by confocal immunofluorescence microscopy, 2) identification GPER1 staining on filamentous structures projecting between the nuclear membrane and the PM, and 3) reciprocal coimmunoprecipitation of GPER1 and CK, including CK7 and CK8. To our knowledge, this is the first observation that a GPCR is capable of reaching and interacting with intermediate filaments. CK7 and CK8 are simple-epithelial ductal-type CKs that are widely distributed and often coexpressed. Little is known about CK7, which usually pairs with CK19, but the related CK8, which pairs with CK18, has been shown to associate with the external leaflet of the PM in cancer cells (Gires et al., 2005). In addition, CKs are necessary for membrane incorporation of glucose transporters 1 and 3 (Vijayaraj et al., 2009). One suggestion is that endocytic vesicles use CK filaments to redistribute GPER1 to unique functional locations in the cell such as cell-cell or cell-

basement membrane contacts (Toivola et al., 2005) via interaction with the adapter complex AP3 involved in clathrin-mediated endocytosis in a way similar to the CK-related proteins vimentin, peripherin, and α-internexin (Styers et al., 2004).

GPER1 expression at the protein level is heterogeneous both in native and recombinant cells, with products observed both below and above the theoretical receptor mass, which may be consequences of detergent-resistant protein complexes, glycosylation, and degradation. The receptor monomer appears to be a 40- to 45-kDa protein as observed in both native and recombinant cells. The relationship of this protein to the 50- to 55-kDa receptor protein is not clear, but the latter may in part be a glycosylated form of the former because it decreased in mass upon PNGase F treatment. The 100-kDa receptor band identified primarily in receptor immunoprecipitates may be a detergent-resistant receptor dimer or a monomer interacting with another protein. It is noteworthy that the 100-kDa band was the primary receptor band enriched in CK immunoprecipitates, suggesting that it is this receptor form that interacts with CK. On the other hand, CK may serve as a scaffold for receptor interactions with other proteins. Some evidence was obtained that N-glycosylation plays a role in receptor interactions, at least for FGPER1, because PNGase treatment increased the amount of the 100-kDa FGPER1 band apparently at the expense of the lower mass bands. One explanation is that N-deglycosylation makes the proteins in the immunoprecipitate more hydrophobic, thus promoting complex formation. On the other hand, N-deglycosylation of the two asparagines in the receptor N-terminal domain may influence the immunoreactivity of the nearby N-terminal FLAG epitope. Apparent receptor degradation products were also observed, which should be cellular because all preparations contained a complete protease inhibitor cocktail. It is interesting to note that CK intermediate filaments associate with proteasomes (Olink-Coux et al., 1994), and ERα is degraded via a ubiquitin-proteasome pathway involving receptor association with CK8 and CK18 (Long and Nephew, 2006).

The localization of GPER1 signaling is also a matter of debate. Using recombinant cell systems and membrane-permeable and -impermeable E2 analogs, GPER1 was proposed to function either in the PM or intracellularly in the ER. PM signaling was based on GPER1 localization in this compartment, stimulation of cAMP production by mem-

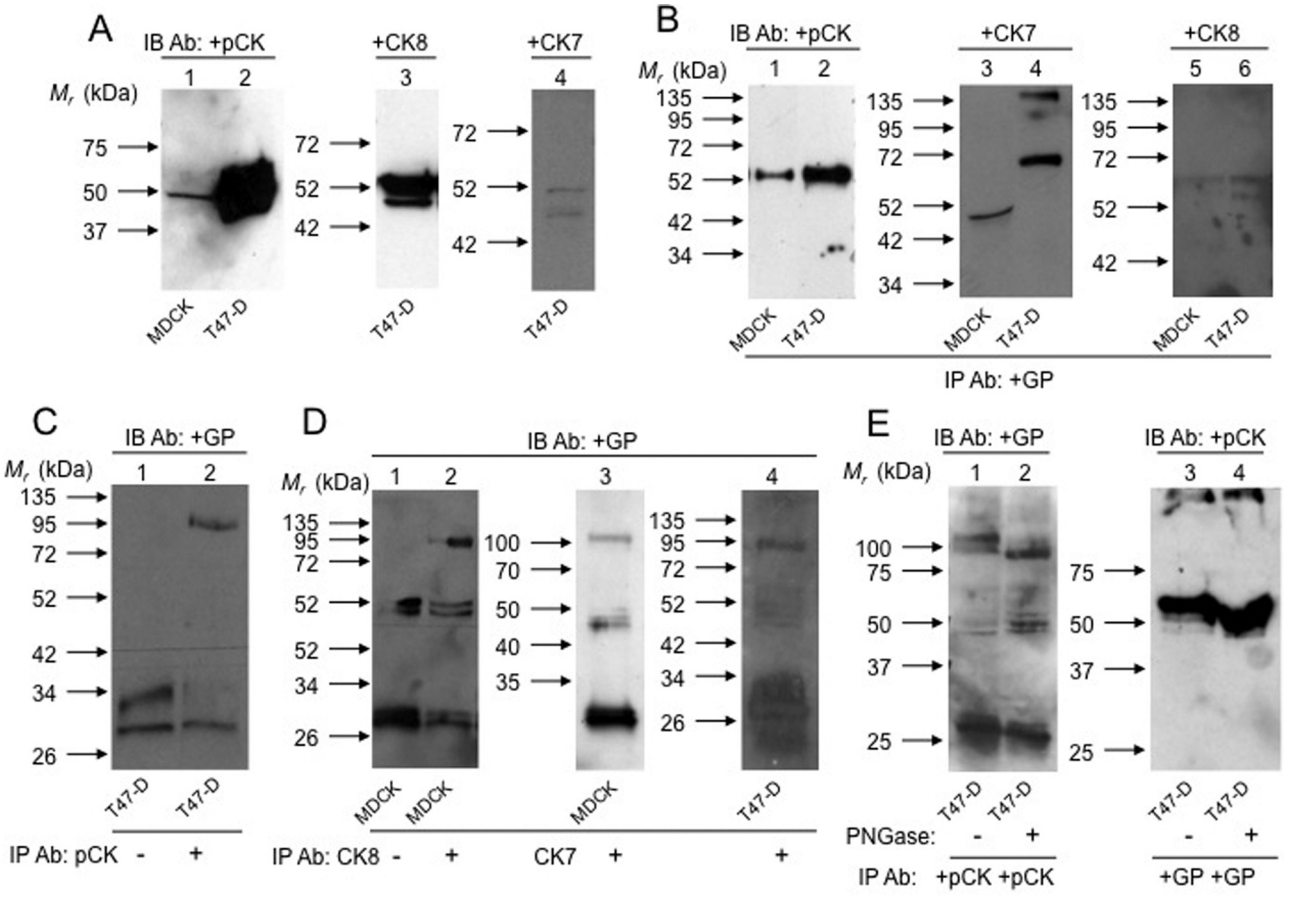

Fig. 7. Native GPER1-CK association. A, MDCK cells (MDCK, lane 1) and T47-D cells (T47-D, lanes 2–4) were lysed and immunoblotted with mouse pan-CK antibody (lanes 1 and 2), mouse CK8 antibody (lane 3), or mouse CK7 antibody (lane 4). B, MDCK cells (MDCK, lanes 1, 3, and 5) and T47-D cells (T47-D, lanes 2, 4, and 6) were lysed, immunoprecipitated with goat GPER1 antibody (GP) precoupled to protein G-Sepharose, and then immunoblotted with pan-CK antibody (lanes 1 and 2), CK7 antibody (lanes 3 and 4) or CK8 antibody (lanes 5 and 6). C, T47-D cells (T47-D) were lysed, immunoprecipitated with protein G-Sepharose without (−, lane) and with precoupled pan-CK antibody (+, lane), and then immunoblotted with GPER1 antibody (GP). D, MDCK cells (MDCK, lanes 1–3) and T47-D cells (T47-D, lane 4) were lysed, immunoprecipitated with CK8 antibody (lanes 1 and 2) or CK7 antibody (lane 3) precoupled to protein G-Sepharose, and then immunoblotted with GPER1 antibody. E, T47-D cells (T47-D) were lysed, immunoprecipitated with pan-CK antibody (lanes 1 and 2) or GPER1 antibody (lanes 3 and 4) precoupled to protein G-Sepharose, respectively, treated without (lanes 1 and 3) or with PNGase F (lanes 2 and 4) and then immunoblotted with GPER1 antibody. Molecular mass (M_r) standards (in kilodaltons) (left side arrows) are indicated. The results are representative of experiments performed at least three times.

brane-impermeable E2 analogs, E2-stimulated PM GTPγS binding, and GPER1-dependent PM E2 binding (Filardo et al., 2002, 2007; Thomas et al., 2005). On the other hand, ER-associated signaling was based on the identification of GPER1 in this compartment in some cells and stimulation of intracellular Ca^{2+} signaling and PI3-kinase translocation only by membrane-permeable E2 analogs (Revankar et al., 2005, 2007).

Here, we show that G-1 and E2 both stimulated GPER1-dependent cAMP production in several native cell lines from several species, which is a PM-defined event. G-1 and E2 also recruited β-arrestin2-GFP to the PM, which is consistent with the receptor for these agonists being localized in this compartment and also a possible mechanism of GPER1-mediated extracellular signal-regulated kinase signaling as shown previously for other receptors (Galandrin and Bouvier, 2006). Thus, we conclude that it is in the PM that GPER1 couples to G_s/adenylate cyclase and β-arrestin2 in native cells. Whether GPER1 is able to couple to other signals in other subcellular compartments such as intermediate filaments remains an open question. It is noteworthy that the efficacy of E2 on GPER1-mediated cAMP production in these cells was only a fraction of that of G-1, which suggests that E2 may act as a partial agonist or G-1 as a superagonist on this response. Although our assay of β-arrestin2 recruitment is only semiquantitative, the efficacies of G-1 and E2 on this response seemed to be approximately equal. Thus, E2 may exhibit biased signaling at GPER1 (i.e., full agonist on β-arrestin2 recruitment and partial agonist on cAMP production), a behavior described for agonists at several other GPCR (Kenakin, 2007), including receptors coupled to both cAMP and β-arrestin signaling (Galandrin and Bouvier, 2006).

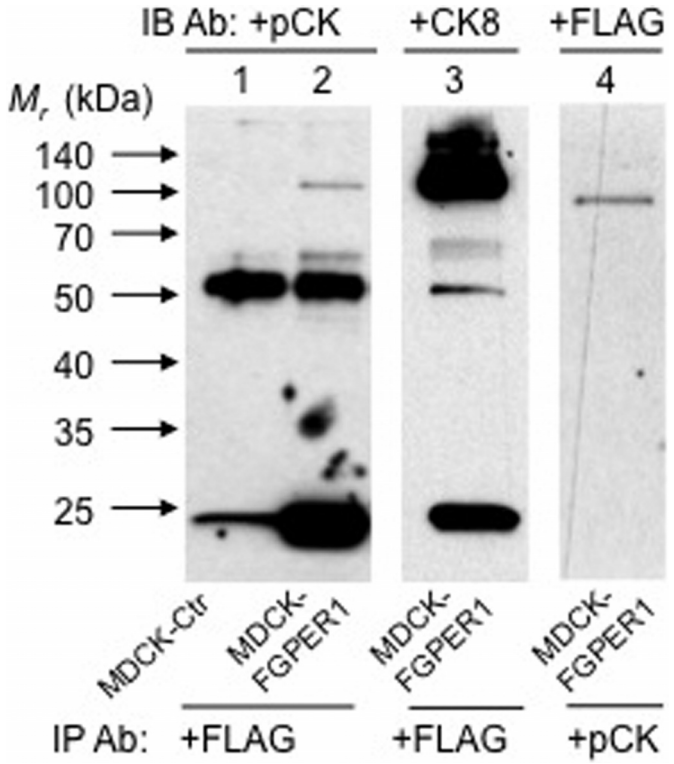

Fig. 8. Recombinant GPER1-CK association. MDCK cells without (MDCK-Ctr.) and with transient transfection of FGPER1 cDNA (MDCK-FGPER1) were lysed, immunoprecipitated with M2 FLAG antibody (lanes 1–3) or pan-CK antibody (lane 4), and immunoblotted with pan-CK antibody (lanes 1 and 2), CK8 antibody (lane 3), or M2 FLAG antibody (lane 4). Molecular mass (M_r) standards (in kilodaltons) (left side arrows) are indicated. The results are representative of experiments performed at least three times.

In summary, we show that GPER1 is localized and signals via cAMP production and β-arrestin2 recruitment in the PM. Receptors also reach CK intermediate filaments. CK is highly expressed in cancer epithelial cells and has long been used to classify cancer subtypes (Moll et al., 2008). Considering that GPER1 influences growth factor signaling pathways (Filardo and Thomas, 2005; Prossnitz et al., 2008) and cancer cell proliferation (Pandey et al., 2009; Ariazi et al., 2010) and that receptor expression is associated with cancer growth (Filardo et al., 2006; Smith et al., 2007), it is tempting to propose that GPER1-CK association in epithelial cells provides an important functional link in this disease.

Acknowledgments

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Authorship Contributions

Participated in research design: Sandén, Broselid, Olde, and Leeb-Lundberg.

Conducted experiments: Sandén, Broselid, Cornmark, Andersson, Mårtensson, and Daszkiewicz-Nilsson.

Performed data analysis: Sandén, Broselid, Olde, and Leeb-Lundberg.

Wrote or contributed to the writing of the manuscript: Sandén, Broselid, and Leeb-Lundberg.

Other: Leeb-Lundberg acquired funding for the research.

References

Ahola TM, Manninen T, Alkio N, and Ylikomi T (2002) G protein-coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells. *Endocrinology* **143:**3376–3384.

Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, Cunliffe HE, Black MA, Donato AL, Arterburn JB, Oprea TI, et al. (2010) The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res* **70:**1184–1194.

Barak LS, Ferguson SS, Zhang J, and Caron MG (1997) A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J Biol Chem* **272:**27497–27500.

Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, Parker MA, Tkachenko SE, Savchuck NP, Sklar LA, et al. (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* **2:**207–212.

Brailoiu E, Dun SL, Brailoiu GC, Mizuo K, Sklar LA, Oprea TI, Prossnitz ER, and Dun NJ (2007) Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. *J Endocrinol* **193:**311–321.

Brothers SP, Janovick JA, and Conn PM (2003) Unexpected effects of epitope and chimeric tags on gonadotropin-releasing hormone receptors: implications for understanding the molecular etiology of hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* **88:**6107–6112.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:**156–159.

Enquist J, Skröder C, Whistler JL, and Leeb-Lundberg LM (2007) Kinins promote B_2 receptor endocytosis and delay constitutive B_1 receptor endocytosis. *Mol Pharmacol* **71:**494–507.

Eriksson JE, Dechat T, Grin B, Helfand B, Mendez M, Pallari HM, and Goldman RD (2009) Introducing intermediate filaments: from discovery to disease. *J Clin Invest* **119:**1763–1771.

Ferrero M, Spyratos F, Desplaces A, Andrieu C, Phillips E, and Rouëssé J (1989) Cytokeratin analysis of breast and leukemia tumor cell lines by flow cytometry. *Biol Cell* **65:**305–308.

Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, and Thomas P (2007) Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. *Endocrinology* **148:**3236–3245.

Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, DeLellis RA, Steinhoff MM, and Sabo E (2006) Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. *Clin Cancer Res* **12:**6359–6366.

Filardo EJ, Quinn JA, Frackelton AR Jr., and Bland KI (2002) Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol* **16:**70–84.
Filardo EJ and Thomas P (2005) GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. *Trends Endocrinol Metab* **16:**362–367.
Funakoshi T, Yanai A, Shinoda K, Kawano MM, and Mizukami Y (2006) G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane. *Biochem Biophys Res Commun* **346:**904–910.
Galandrin S and Bouvier M (2006) Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* **70:**1575–1584.
Gires O, Andratschke M, Schmitt B, Mack B, and Schaffrik M (2005) Cytokeratin 8 associates with the external leaflet of plasma membranes in tumour cells. *Biochem Biophys Res Commun* **328:**1154–1162.
Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Ström A, Treuter E, Warner M, et al. (2007) Estrogen receptors: how do they signal and what are their targets. *Physiol Rev* **87:**905–931.
Heuser JE and Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol* **108:**389–400.
Kenakin T (2007) Functional selectivity through protean and biased agonism: who steers the ship? *Mol Pharmacol* **72:**1393–1401.
Kolkova Z, Noskova V, Ehinger A, Hansson S, and Casslén B (2010) G protein-coupled estrogen receptor 1 (GPER, GPR 30) in normal human endometrium and early pregnancy decidua. *Mol Hum Reprod* **16:**743–751.
Kotarsky K, Owman C, and Olde B (2001) A chimeric reporter gene allowing for clone selection and high-throughput screening of reporter cell lines expressing G-protein-coupled receptors. *Anal Biochem* **288:**209–215.
Lin BC, Suzawa M, Blind RD, Tobias SC, Bulun SE, Scanlan TS, and Ingraham HA (2009) Stimulating the GPR30 estrogen receptor with a novel tamoxifen analogue activates SF-1 and promotes endometrial cell proliferation. *Cancer Res* **69:**5415–5423.
Long X and Nephew KP (2006) Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor-alpha. *J Biol Chem* **281:**9607–9615.
Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, Montanaro D, Musti AM, Picard D, and Andò S (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. *J Biol Chem* **279:**27008–27016.
Mårtensson UE, Salehi SA, Windahl S, Gomez MF, Swärd K, Daszkiewicz-Nilsson J, Wendt A, Andersson N, Hellstrand P, Grände PO, et al. (2009) Deletion of the G protein-coupled receptor GPR30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. *Endocrinology* **150:**687–698.
Matsuda K, Sakamoto H, Mori H, Hosokawa K, Kawamura A, Itose M, Nishi M, Prossnitz ER, and Kawata M (2008) Expression and intracellular distribution of the G protein-coupled receptor 30 in rat hippocampal formation. *Neurosci Lett* **441:**94–99.
Meier KE, Snavely MD, Brown SL, Brown JH, and Insel PA (1983) Alpha 1- and beta 2-adrenergic receptor expression in the Madin-Darby canine kidney epithelial cell line. *J Cell Biol* **97:**405–415.
Moll R, Divo M, and Langbein L (2008) The human keratins: biology and pathology. *Histochem Cell Biol* **129:**705–733.
Olde B and Leeb-Lundberg LM (2009) GPR30/GPER1: searching for a role in estrogen physiology. *Trends Endocrinol Metab* **20:**409–416.
Olink-Coux M, Arcangeletti C, Pinardi F, Minisini R, Huesca M, Chezzi C, and Scherrer K (1994) Cytolocation of prosome antigens on intermediate filament subnetworks of cytokeratin, vimentin and desmin type. *J Cell Sci* **107:**353–366.
Otto C, Rohde-Schulz B, Schwarz G, Fuchs I, Klewer M, Brittain D, Langer G, Bader B, Prelle K, Nubbemeyer R, et al. (2008) G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol. *Endocrinology* **149:**4846–4856.
Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M, and Picard D (2009) Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. *EMBO J* **28:**523–532.
Pollack V, Scheiber K, Pfaller W, and Schramek H (1997) Loss of cytokeratin expression and formation of actin stress fibers in dedifferentiated MDCK-C7 cell lines. *Biochem Biophys Res Commun* **241:**541–547.
Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, and Hathaway HJ (2008) Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol* **70:**165–190.
Razandi M, Pedram A, Merchenthaler I, Greene GL, and Levin ER (2004) Plasma membrane estrogen receptors exist and functions as dimers. *Mol Endocrinol* **18:**2854–2865.
Revankar CM, Cimino DF, Sklar LA, Arterburn JB, and Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307:**1625–1630.
Revankar CM, Mitchell HD, Field AS, Burai R, Corona C, Ramesh C, Sklar LA, Arterburn JB, and Prossnitz ER (2007) Synthetic estrogen derivatives demonstrate the functionality of intracellular GPR30. *ACS Chem Biol* **2:**536–544.
Sakamoto H, Matsuda K, Hosokawa K, Nishi M, Morris JF, Prossnitz ER, and Kawata M (2007) Expression of G protein-coupled receptor-30, a G protein-coupled membrane estrogen receptor, in oxytocin neurons of the rat paraventricular and supraoptic nuclei. *Endocrinology* **148:**5842–5850.
Schweizer J, Bowden PE, Coulombe PA, Langbein L, Lane EB, Magin TM, Maltais L, Omary MB, Parry DA, Rogers MA, et al. (2006) New consensus nomenclature for mammalian keratins. *J Cell Biol* **174:**169–174.
Smith HO, Leslie KK, Singh M, Qualls CR, Revankar CM, Joste NE, and Prossnitz ER (2007) GPR30: a novel indicator of poor survival for endometrial carcinoma. *Am J Obstet Gynecol* **196:**386.e1–e11.
Styers ML, Salazar G, Love R, Peden AA, Kowalczyk AP, and Faundez V (2004) The endo-lysosomal sorting machinery interacts with the intermediate filament cytoskeleton. *Mol Biol Cell* **15:**5369–5382.
Thomas P, Pang Y, Filardo EJ, and Dong J (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* **146:**624–632.
Toivola DM, Tao GZ, Habtezion A, Liao J, and Omary MB (2005) Cellular integrity plus: organelle-related and protein-targeting functions of intermediate filaments. *Trends Cell Biol* **15:**608–617.
Vijayaraj P, Kröger C, Reuter U, Windoffer R, Leube RE, and Magin TM (2009) Keratins regulate protein biosynthesis through localization of GLUT1 and -3 upstream of AMP kinase and Raptor. *J Cell Biol* **187:**175–184.
Whistler JL, Enquist J, Marley A, Fong J, Gladher F, Tsuruda P, Murray SR, and Von Zastrow M (2002) Modulation of postendocytic sorting of G protein-coupled receptors. *Science* **297:**615–620.
Windahl SH, Andersson N, Chagin AS, Mårtensson UE, Carlsten H, Olde B, Swanson C, Movérare-Skrtic S, Sävendahl L, Lagerquist MK, et al. (2009) The role of the G protein-coupled receptor GPR30 in the effects of estrogen in ovariectomized mice. *Am J Physiol Endocrinol Metab* **296:**E490–E496.

Address correspondence to: Dr. L. M. Fredrik Leeb-Lundberg, Department of Experimental Medical Science, Lund University, BMC A12, SE-22184 Lund, Sweden. E-mail: fredrik.leeb-lundberg@med.lu.se